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Igf3 activates β-catenin signaling to stimulate spermatogonial differentiation in zebrafish

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Abstract

Follicle-stimulating hormone (Fsh) is a major regulator of spermatogenesis, targeting somatic cell functions in the testes. We reported previously that zebrafish Fsh promoted the differentiation of type A undifferentiated spermatogonia (A_{und}) by stimulating the production of factors that advance germ cell differentiation, such as androgens, insulin-like peptide 3 (Insl3) and insulin-like growth factor 3 (Igf3). In addition, Fsh also modulated the transcript levels of several other genes, including some belonging to the Wnt signaling pathway. Here, we evaluated if and how Fsh utilizes part of the canonical Wnt pathway to regulate the development of spermatogonia. We quantified the proliferation activity and relative section areas occupied by A_{und} and type A differentiating (A_{diff}) spermatogonia and we analyzed the expression of selected genes in response to recombinant proteins and pharmacological inhibitors. We found that from the three downstream mediators of Fsh activity we examined, Igf3, but not 11-ketotestosterone or Insl3, modulated the transcript levels of two β-catenin sensitive genes (*cyclinD1* and *axin2*). Using a zebrafish β-catenin signaling reporter line, we showed that Igf3 activated β-catenin signaling in type A spermatogonia and that this activation did not depend on the release of Wnt ligands. Pharmacological inhibition of the β-catenin or of the phosphoinositide 3-kinase (PI3K) pathways revealed that Igf3 activated β-catenin signaling in a manner involving PI3K to promote the differentiation of A_{und} to A_{diff} spermatogonia. This mechanism represents an intriguing example for a pituitary hormone like Fsh using Igf signaling to recruit the evolutionary conserved, local β-catenin signaling pathway to regulate spermatogenesis.

Introduction

Long-term spermatogenesis relies on a population of undifferentiated germ cells including spermatogonial stem cells (SSCs). They reside in a specific microenvironment, the SSC niche, formed by somatic cells such as Sertoli cells (SCs), Leydig cells (LCs) and myoid cells, which provide SSCs with signals relevant to modulate their behavior (Oatley *et al*. 2009, Ding *et al*. 2011, Chen *et al*. 2016a, Lord & Oatley 2017, De Rooij 2017). SSCs can be quiescent or proliferate either to self-renew thereby producing more SSCs or undergo a differentiating proliferation to eventually produce spermatozoa. A balanced self-renewal/differentiation activity is critical to maintain long-term spermatogenesis and testis tissue homeostasis (De Rooij 2015, 2017).

The pituitary gonadotropin Fsh is a major regulator of spermatogenesis. In the mammalian testis, the *FSH receptor* gene *(FSHR)* is expressed exclusively by SCs. FSH stimulates the production of growth factors important for spermatogonial proliferation and differentiation, such as FGF2 or IGF1 (Mullaney & Skinner 1992, Pitetti *et al.* 2013). In fish, the androgen-producing LCs also express the *fshr* gene and Fsh is a potent steroidogenic hormone (Ohta *et al.* 2007, García-López *et al.* 2010). Similar to other vertebrates, androgens stimulate spermatogenesis in fish (Miura *et al.* 1991, Leal *et al.* 2009). However, zebrafish Fsh also stimulates spermatogonial differentiation in an androgen-independent manner, for example by stimulating the release of Igf3 mainly from SCs (Nóbrega *et al.* 2015) and of Insl3 from LCs (Assis *et al.* 2016, Crespo *et al.* 2016). Moreover, Fsh suppresses the production of the SC-derived anti-Müllerian hormone (Amh) (Skaar *et al.* 2011), which would otherwise exert inhibitory effects on spermatogenesis (Morais *et al.* 2017).

Crespo *et al.* (2016) identified ~200 testicular transcripts responding to zebrafish Fsh in an androgen-independent manner, including genes belonging to the Wnt signaling pathway. Similar results were reported for rainbow trout testis tissue (Sambroni *et al.* 2013). The Wnt signaling system is a conserved cell-to-cell communication system that consists of canonical and non-canonical pathways. The former is also referred to as β-catenin-dependent pathway and relies on inhibiting the β-catenin destruction complex (Salic *et al.* 2000), thereby increasing the levels of cytoplasmic β-catenin (Nusse 1999). After its translocation into the nucleus, β-catenin interacts with transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family, inducing the expression of Wnt reporter genes such as *cyclinD1* and *axin2* (Tetsu & McCormick 1999, Shtutman *et al.* 1999, Jho *et al.* 2002, Van Amerongen *et al.* 2012, Takase & Nusse 2016).

The β-catenin-dependent pathway modulates spermatogenesis in mammals (Yeh *et al.* 2012, Kerr *et al.* 2014, Takase & Nusse 2016, Tokue *et al.* 2017), but studying mice carrying *β-catenin* mutations provided inconsistent results. Kerr *et al.* (2014) reported that loss of *β-catenin* in all germ cells depleted spermatocytes and spermatids without affecting the number of PLZF-positive (a marker for undifferentiated spermatogonia) germ cells. In contrast, when *β-catenin* knockout was mediated by *Stra8*-Cre (expressed by differentiating spermatogonia), mutant mice showed normal spermatogenesis (Rivas *et al.* 2014). Still, a later report described that the canonical WNT reporter gene *Axin2* is expressed specifically in undifferentiated spermatogonia and that *β-catenin* knockout in

Axin2-positive spermatogonia reduced proliferation activity and number of PLZF-positive spermatogonia in mice, resulting in the loss of differentiating spermatogonia (Takase & Nusse 2016). Moreover, inhibiting WNT secretion by knocking out *Gpr177* (the mouse orthologue of *Drosophila wls*) that codes for a WNT ligand chaperone critical for exocytosis, either in germ cells or in SCs, had no or limited effects on spermatogenesis (Chen *et al.* 2016), challenging previous conclusions.

In the present study, we investigated if canonical Wnt signaling participates in mediating stimulatory effects of Fsh on zebrafish spermatogenesis, using a primary testis tissue culture system. We report for the first time in a vertebrate that Fsh recruits the β-catenin signaling system via Igf signaling to regulate germ cell development.

Materials and methods

Animals

Adult WT male zebrafish (AB strain) and *Tg(7xTCF-Xla.Siam:GFP)ia4* male zebrafish (Moro *et al.* 2012) between 4 and 12 months of age were used in this study. All experiments followed the Dutch National regulations for animal care. The experimental protocols were approved by the Utrecht University Experimental Animal Committee (2013.III.06.045 and NVWA 10800).

Tissue culture

To study the role of the canonical Wnt signaling system, adult zebrafish testes were dissected for tissue culture experiments using a previously described system (Leal *et al.* 2009), in which one testis was incubated under control conditions and the other testis under experimental conditions.

We have reported previously that 100 ng/mL Fsh increased the proliferation of both A_{und} and A_{diff} spermatogonia. Since Fsh also elevated androgen production (García-Lopez *et al.* 2010) and the production of the growth factors Insl3 and Igf3 (Nóbrega *et al.* 2015, Assis *et al.* 2016, Crespo *et al.* 2016), we studied if androgen, Insl3 or Igf3 use the β-catenin-dependent pathway to stimulate spermatogenesis. To this end, zebrafish testes were incubated under basal conditions (Leal *et al.* 2009) or in the presence of 11-ketotestosterone (11-KT; Sigma; 200 nM in 0.01% ethanol; García-López *et al.* 2010) of Insl3 (100 ng/mL; Crespo *et al.* 2016) or of Igf3 (100 ng/mL; Nóbrega *et al.* 2015) for 4 or 5 days.

In a parallel experiment, zebrafish testes were incubated for 5 days in the presence of Fsh (100 ng/mL) with or without NVP-AEW541 (10 μM in medium containing 0.1% DMSO; Selleckchem; Morais *et al.* 2013), an Igf1 receptor inhibitor. The production of biologically active steroids was blocked by including trilostane (25 μg/mL in medium containing 0.1% dimethyl sulfoxide; Chemos), an inhibitor of 3β-hydroxysteroid dehydrogenase activity, in all experiments with Fsh, a potent steroidogenic hormone in zebrafish (Garcia-Lopez *et al.* 2010).

To study if Igf3 directly stimulates β-catenin signaling, zebrafish testes were incubated for 5 days under basal conditions or in the presence of Igf3 (100 ng/mL) or in the presence of Igf3 (100 ng/mL) with or without 50 μM IWP-12 (Sigma-Aldrich) in medium containing 0.05% DMSO. IWP-12 inhibits an enzyme required to release Wnt ligands (porcupine) in zebrafish (Chen *et al.* 2009, Dodge *et al.* 2012). Since no statistical differences were found as regards BrdU incorporation ($P=0.718$ for A_{und} and $P=0.951$ for A_{diff}; unpaired Student's *t*-test) and proportion of area ($P=0.563$ for A_{und} and $P=0.595$ for A_{diff}; unpaired Student's *t*-test) after incubation with Igf3 in both experiments, we treated them as a single group to compare three different conditions (basal, Igf3 and Igf3 with IWP-12).

To study Igf3 effects on β-catenin signaling, zebrafish testes were incubated for 5 days under basal conditions (Leal *et al.* 2009) or in the presence of Igf3 (100 ng/mL). In parallel experiment, testes were incubated in the presence of Igf3 (100 ng/mL) with or without 10 μM XAV939 (Sigma-Aldrich) (Shimizu *et al.* 2012) or 12 μM IWR-1 (Sigma-Aldrich) (Shimizu *et al.* 2012, Wehner *et al.* 2014) in medium containing 0.01% or 0.012% DMSO, respectively. XAV939 and IWR-1 are compounds inhibiting β-catenin signaling by increasing the stability of Axin, a key protein of the β-catenin destruction complex, thereby decreasing the levels of cytoplasmatic β-catenin (Huang *et al.* 2009, Shimizu *et al.* 2012). Since no statistical differences were found as regards BrdU incorporation and proportion of area (one-way ANOVA, followed by Tukey's *post hoc* test) occupied by type A spermatogonia after incubation with Igf3 in the three experiments, we treated them as a single group to compare four different conditions (basal, Igf3 and Igf3 with XAV939 or IWR-1).

To further characterize the effect of XAV939 on Igf3-stimulated spermatogenesis, testes were incubated in the presence of Igf3 (100 ng/mL) alone or in the additional presence of 10 μM XAV939 for 5 days to analyze the expression of selected genes.

Igf1 receptor activation mainly triggers the MAPK pathway and the PI3K pathway (LeRoith 2008).

To study which mechanism Igf3 uses to stimulate β-catenin signaling, we examined if a MAPK inhibitor (50 μM PD98059 (InvivoGen) in medium containing 0.05% DMSO; Nóbrega *et al.* 2015) or a PI3K inhibitor (20 μM LY294002 (Sigma-Aldrich) in medium containing 0.02% DMSO; Pozios *et al.* 2001) modulated Igf3-induced changes in spermatogenesis or in the transcript levels of two β-catenin-sensitive genes or four germ cell markers, following an incubation period of 5 days.

In case of the 5-day incubation experiment, the medium was changed once after 3 days of incubation. Testis tissue derived from the experiment described above was fixed for morphological analysis or snap-frozen in liquid nitrogen at the end of the incubation period and stored at −80°C until RNA extraction.

Transcript levels

The relative transcript levels of germ cell-marker genes and other genes of interest (Table 1) were analyzed by real-time, quantitative polymerase chain reaction (qPCR) assays.

Total RNA was isolated from the tissue using an RNAqueous Micro kit (Ambion), according to the manufacturer's protocol. cDNA synthesis from total RNA and quantification of transcript levels were carried out as described previously (Bogerd *et al.* 2001). In brief, 2 μg of total RNA were reverse transcribed using 250 U of Superscript II RNase⁻ reverse transcriptase (Life Technologies). qPCRs were performed in SYBR Green assay mix (Applied Biosystems), specific qPCR primers (900 nM each) and 5 μL cDNA in a total volume of 20 μL. The quantification cycle (Cq) values were determined in a ViiA7 Real-Time PCR System (Applied Biosystems) using default settings. The relative amounts of mRNA in the cDNA samples were calculated using the arithmetic comparative method (ΔΔCt method; Livak & Schmittgen 2001), as described in Bogerd *et al.* (2001). Transcript levels of the *elongation factor 1a* (*ef1a*) were stable (inset in Fig. 1A and B, 3I, and 4C and D) and therefore, *ef1a* was used to normalize gene expression. All results were expressed as fold induction with respect to the control group.

Quantification of spermatogonial proliferation and of proportions of section surface areas

To quantify the proliferation activity of A_{und} and A_{diff} spermatogonia, 100 μg/mL of the proliferation marker 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) was

Table 1 Primers used for gene expression studies (Fw = forward; Rv = reverse) and to generate DNA templates for DIG-labeled cRNA probe syntheses for *in situ* hybridization.

Target genes	Primers name	Sequence (5′-3′)	Gene information
elf1a	2476 (Fw)	GCCGTCCCACCGACAAG	Reference gene (Morais *et al.* 2013)
	2477 (Rv)	CCACACGACCCACAGGTACAG	
cyclinD1	5231 (Fw)	CAAGCAGATACTGCGCAAACA	This paper
	5232 (Rv)	GGGCTTGCGATGAAGTTGAC	
axin2	4615 (Fw)	ACCCTCGGACACTTCAAGGAA	This paper
	4616 (Rv)	TCACTGGCCCTTTTGAAGAAGTAT	
foxa2	5741 (Fw)	GTCAAAATGGAGGGACACGAAC	Potential marker for type A undifferentiated spermatogonia (Safian *et al.* 2017)
	5743 (Rv)	CATGTTGCTGACCGAGGTGTAA	
nanos2	4817 (Fw)	AAACGGAGAGACTGCGCAGAT	Expressed in type A_{und} spermatogonia (Beer *et al.* 2013, Bellaiche *et al.* 2014)
	4818 (Rv)	CGTCCGTCCCTTGCCTTT	
piwil1	2542 (Fw)	GATACCGCTGCTGGAAAAAGG	Expressed in all generations of type A spermatogonia (Houwing *et al.* 2008)
	2543 (Rv)	TGGTTCTCCAAGTGTGTCTTGC	
piwil2	2994 (Fw)	TGATACCAGCAAGAAGAGCAGATCT	Expressed in all germ cell type except type A_{und} and spermatozoa (Houwing *et al.* 2008)
	2995 (Rv)	ATTTGGAAGGTCACCCTGGAGTA	
dazl	3104 (Fw)	AGTGCAGACTTTGCTAACCCTTATGTA	Expressed by B spermatogonia and primary spermatocytes (Chen *et al.* 2013)
	3105 (Rv)	GTCCACTGCTCCAAGTTGCTCT	
igf1ra	4982 (Fw)[a]	T3Rpp-TACACACATTTCCCTCCTCCCTTTGTCT	Igf1 receptor a (This paper)
	4984 (Rv)[b]	T7Rpp-TGGATTAACCAGTAACATGAGCAATCACTCA	
igf1rb	4983 (Fw)[a]	T3Rpp-ATGCGTCGGATGTGTGTCAAGCCACT	Igf1 receptor b (This paper)
	4985 (Rv)[b]	T7Rpp-CAGATGCAAGATTTCTCCTTCACCACAGATGA	

[a]Primer 4982 and 4983 contain the T3 RNA polymerase promoter sequence (underlined) at its 5′-end (T3Rpps; 5′-GGGCGGGTGTTATTAACCCTCACTAAAGGG-3′); [b]Primer 4984 and 4985 contain the T7 RNA polymerase promoter sequence (underlined) at its 5′-end (T7Rpps; 5′-CCGGGGGGTGTAATACGACTCACTATAGGG-3′).

added to the tissue culture medium during the last 6 h of the incubation period. After fixation in methacarn (60% (v/v) absolute ethanol, 30% chloroform and 10% acetic acid), the samples were dehydrated in graded ethanol (70, 96 and 100%), embedded in Technovit 7100 (Heraeus Kulzer) and sectioned at a thickness of 4 μm. To determine the proliferation activity, one set of sections was used to localize BrdU as described previously (Leal *et al.* 2009). The BrdU labeling index was determined by analyzing 100 cysts (A_{diff} spermatogonia) or 100 A_{und} cells, discriminating between BrdU-positive and -negative cysts and cells, respectively. In addition, the number of BrdU-positive 'free' SCs (apparently not associated with germ cells), SCs contacting BrdU-positive and -negative A_{und}, and SCs contacting BrdU-positive and -negative A_{diff} was determined using ten randomly chosen, non-overlapping fields at ×400 magnification.

To quantify the proportion of section area occupied by type A spermatogonia, another set of sections was stained with toluidine blue and 10 randomly chosen, non-overlapping fields were photographed at ×400 magnification with a digital camera. The images were analyzed quantitatively based on the number of points counted over the germ cell types investigated (A_{und} and A_{diff}), using the ImageJ freeware (National Institute of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij) with a 540-point grid, i.e. 5400 data points were analyzed per individual.

Immunofluorescence on paraffin sections

To detect β-catenin-dependent pathway activation, testes from a transgenic zebrafish line (*Tg(7xTCF-Xla.Siam:GFP)[ia4]*) expressing *gfp* upon β-catenin activation were used (Moro *et al.* 2012). Testes from this line were incubated in basal medium or in the presence of Igf3 (100 ng/mL), IWP-12 (50 μM) or Igf3 (100 ng/mL) in combination with XAV939 (10 μM) or IWP-12 (50 μM). After fixation in phosphate-buffered 4% paraformaldehyde at 4°C overnight, the tissue was dehydrated and embedded in paraplast (Sigma-Aldrich). Sections of 5 μm thickness were dewaxed and rehydrated, according to conventional techniques. After antigen retrieval (10 mM sodium citrate, 0.05% Tween 20, pH 6.0; at 98°C for 10 min) non-specific antibody binding was blocked by pre-incubating slides
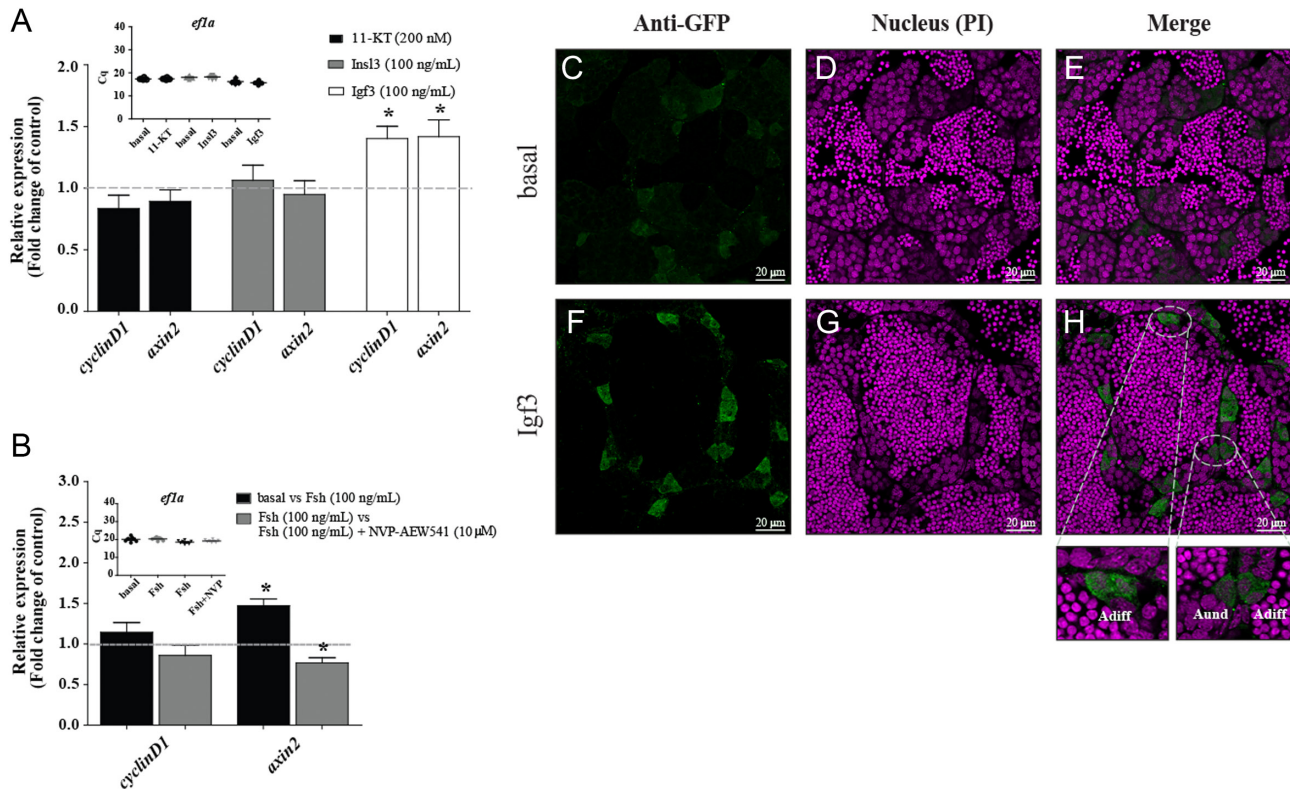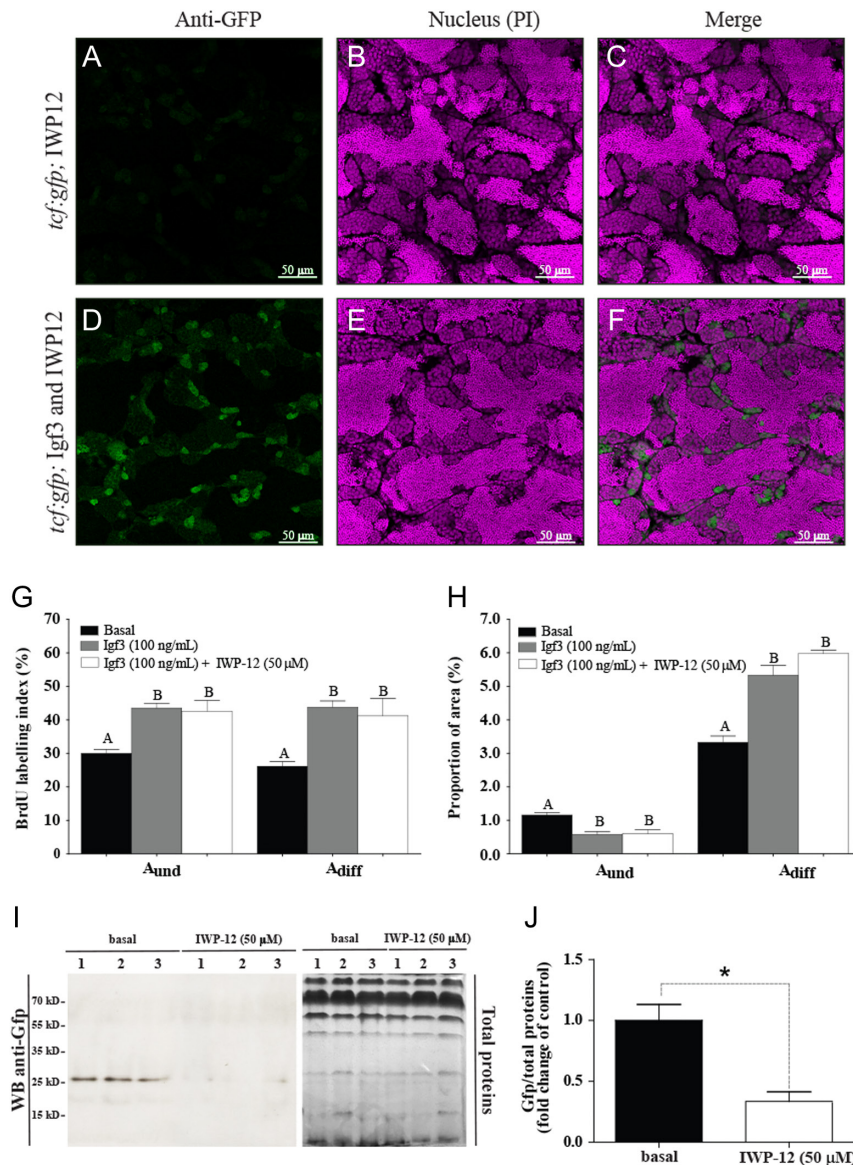
Figure 1

Igf3 activates β-catenin-dependent signaling in type A spermatogonia. (A) Transcript levels of two β-catenin sensitive genes (*cyclinD1* and *axin2*) in basal conditions (dotted line; control condition) or in the presence of 11-KT (200 nM, sample size (*n* = 6), Insl3 (100 ng/mL, *n* = 10) or Igf3 (100 ng/mL, *n* = 7), represented by bars. (B) Transcript levels of *cyclinD1* and *axin2* in the presence of Fsh (100 ng/mL) (dotted line; control condition) or in combination with the inhibitor of Igf1r NVP-AEW541 (10 μM, *n* = 7), represented by bars. The quantification cycles (Cq) of the reference gene (*elf1a*) are shown in the inset. Results are presented as fold change with respect to the control group (basal or 100 ng/mL Fsh, respectively) Asterisks indicate significant differences (*P* < 0.05) to the respective control group. (C, D, E, F, G and H) Immunocytochemical detection of Gfp in testis sections from transgenic *(7x*TCF-Xla. Siam*:GFP)^{ia4}* zebrafish incubated for 3 h under basal conditions or in the presence of Igf3 (100 ng/mL, *n* = 3). A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0124.

with 5% normal goat serum (Sigma-Aldrich) and 1% acetylated bovine serum albumin (BSA; Sigma-Aldrich) at room temperature for 30 min. Then, sections were incubated with a primary antibody raised in rabbit against GFP (Nóbrega *et al.* 2015; 1 μg/mL; TP401, Torrey Pines Biolabs Inc.) in PBS containing 1% BSA at 4°C overnight. After PBS washes, sections were incubated with secondary goat anti-rabbit Alexa Fluor 488 (8 μg/mL, A11034, Sigma-Aldrich) containing 10% normal horse serum (Sigma-Aldrich) at room temperature for 1 h and 30 min. After PBS washes, the sections were incubated for 3 min with propidium iodide (PI; 1 μg/mL, Sigma-Aldrich). Sections were mounted in Vectashield (H-1000, Vector) and images were taken using a confocal laser scanning microscope (LSM 700, Zeiss). Images were acquired and processed using the same settings (pinhole size: 1 AU, same laser power: 12, and gain: 550 mV for Alexa 488 and 400 mV for PI) in all experiments analyzed.

Gfp quantification by Western blot

To detect Wnt ligand release inhibition in response to IWP-12, testes from a transgenic zebrafish line (*Tg(7x*TCF-Xla.Siam*:GFP)^{ia4}*) were incubated under basal conditions or in the presence of IWP-12 (50 μM) for 3 days. After the incubation period, tissue was processed for Western blot analysis to detect Gfp protein production. Proteins were extracted according to Skaar *et al.* (2011) and protein concentrations were determined in a Bradford protein assay (BIO-RAD). Whole testis protein (30 μg) was resolved in 10% SDS-PAGE and Western blot assays were performed according to Fuentes *et al.* (2013) with minor modifications. Briefly, proteins were transferred to polyvinylidene difluoride membranes (Millipore) and blocked for 1 h at room temperature in 5% skimmed milk powder (Millipore) in PBS. Primary GFP antibody incubation was carried out overnight at 4°C (2 μg/mL;

Figure 2

Igf3 activates β-catenin-dependent signaling in a Wnt ligand-independent manner. (A, B, C, D, E and F) Immunocytochemical detection of Gfp in testis sections from transgenic *(*7xTCF-Xla. Siam*:GFP)*[ia4] zebrafish incubated for 3 h in the presence of IWP-12 (50 μM) alone, or in combination with 100 ng/mL Igf3 ($n=3$). Nuclear DNA was stained with propidium iodide (PI). (G) BrdU-labeling indices of type A_{und} and type A_{diff} spermatogonia in basal conditions ($n=7$) or in the presence of Igf3 (100 ng/mL, $n=12$) with or without IWP-12 (50 μM, $n=5$) after 5 days of incubation. (H) Proportion of area occupied by type A_{und} and type A_{diff} spermatogonia in basal conditions ($n=7$) or in the presence of Igf3 (100 ng/mL, $n=12$) with or without IWP-12 (50 μM, $n=5$) after 5 days of incubation. (I) Western blot and (J) densitometric analysis of Gfp production in testis sections from transgenic *(*7xTCF-Xla. Siam*:GFP)*[ia4] zebrafish incubated for 3 days in basal conditions or in the presence of IWP-12 (50 μM) ($n=3$). Letters and asterisk indicate significant differences ($P<0.05$) among groups. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0124.

TP401, Torrey Pines Biolabs Inc.). After PBS washes, secondary horseradish peroxidase-conjugated goat anti-rabbit (0.2 μg/mL; 111-035-003, Jackson ImmunoResearch Laboratories Inc.) was incubated for 1 h at room temperature. Protein detection was performed using Pierce ECL Plus Substrate (Thermo Scientific) according to the manufacturer's instructions. Films were scanned and densitometric analysis of the bands was performed with ImageJ freeware. Gfp analysis was normalized to Coomassie blue staining of total protein.

In situ hybridization on zebrafish testis cryosections

To identify the cell types responding to Igf3, WT zebrafish testes were used to study the expression of *igf1 receptor* *a* (*igf1ra*) and *b* (*igf1rb*) by *in situ* hybridization on cryosections as described previously (Assis *et al.* 2016). For the production of zebrafish *igf1ra* and *igf1rb* DIG-labeled riboprobes (see primers in Table 1), we followed a procedure as reported by Good-Ávila *et al.* (2009).

Statistical analysis

Statistical analyses were carried out using the GraphPad Prism 5 software package. Since our tissue culture system compares in most experiments the two testes of a given fish incubated under control vs experimental conditions, we applied Student's *t*-test for paired observation to estimate the statistical significance of fold changes between treated and control conditions. These data are presented as fold of basal (mean ± standard error of the mean (s.e.m.)).
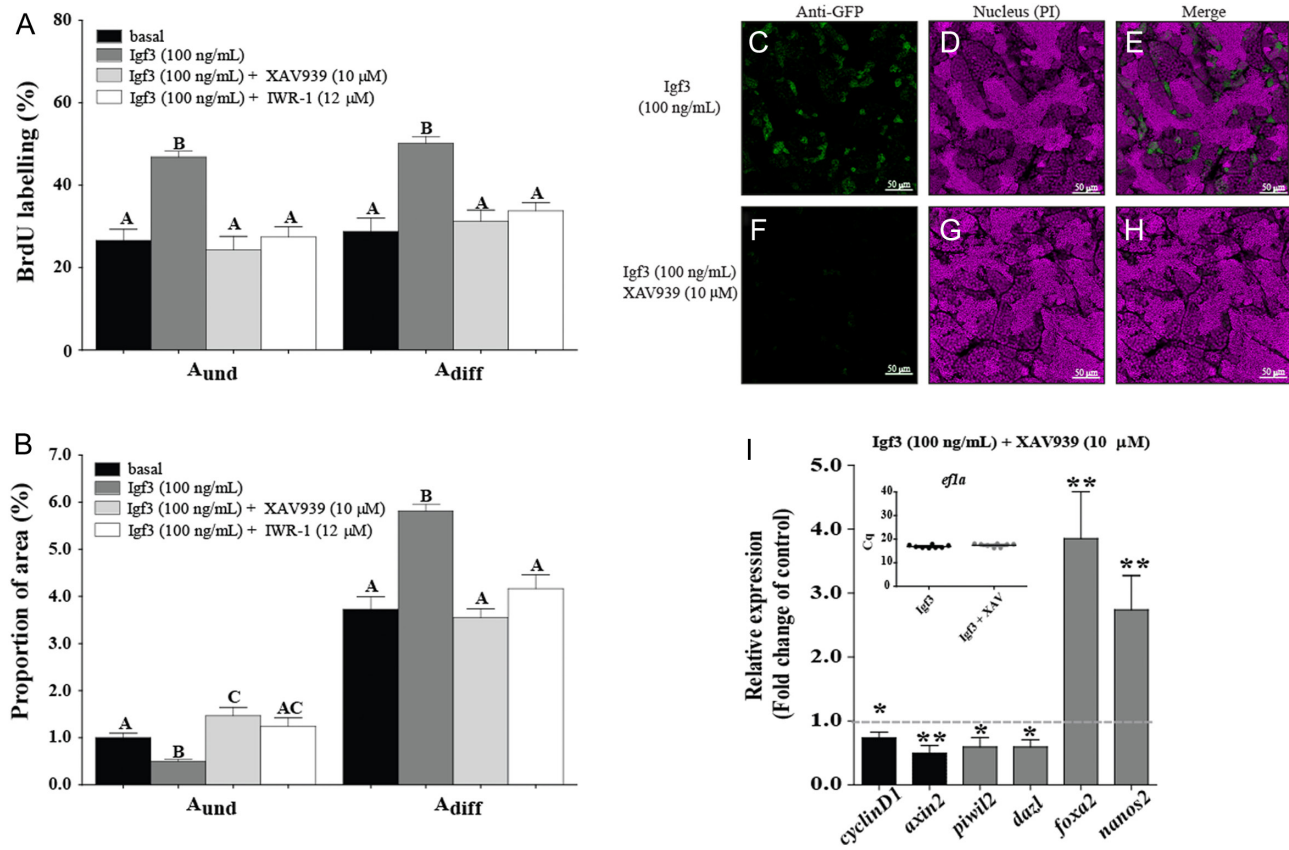
Figure 3

Igf3 uses β-catenin signaling to stimulate the differentiation of type A spermatogonia. (A) BrdU incorporation of type A_{und} and type A_{diff} spermatogonia in basal conditions ($n=6$), in the presence of Igf3 (100 ng/mL) alone ($n=21$), or in the additional presence of either XAV939 (10 μM; $n=7$) or IWR-1 (12 μM; $n=8$). (B) Proportion of section surface area occupied by cysts containing type A_{und} and type A_{diff} spermatogonia in basal conditions ($n=5$), in the presence of Igf3 (100 ng/mL) alone ($n=21$), or in the additional presence of XAV939 (10 μM; $n=7$) or of IWR-1 (12 μM; $n=9$). (C, D, E, F, G and H) Immunofluorescent detection of Gfp in sections of zebrafish testis in the presence of Igf3 (100 ng/mL) alone (C, D and E) or in combination with XAV939 (10 μM; F, G and H) after 3 h of incubation ($n=3$). Nuclear DNA was stained with propidium iodide (PI). (I) Gene expression analysis of two β-catenin sensitive genes (*cyclinD1* and *axin2*) and germ cell marker genes in adult zebrafish testis after 5 days of tissue culture in the presence of Igf3 (100 ng/mL; dotted line; control condition) alone or in combination with 10 μM XAV939 (represented by bars; $n=8$). The quantification cycles (Cq) of the reference gene (*elf1a*) are shown in the inset. Results are presented as percentage (A and B) or as fold changes with respect to the control group (100 ng/mL Igf3) (I). Letters indicate significant differences among groups ($P<0.05$), asterisks (*$P<0.05$; **$P<0.01$) indicate significant differences to the control condition. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0124.

In Figs 2G and H and 3A and B, three and four conditions respectively were compared, so that fold-changes cannot be calculated. In these cases, the BrdU indices (Figs 2G and 3A) or proportion of area values (Figs 2H and 3B) were processed statistically using one-way ANOVA, followed by Tukey's *post hoc* test.

Results

The Fsh-modulated Igf3 stimulates the activation of β-catenin signaling in type A spermatogonia

We have found that Fsh modulated the expression of Wnt-related genes (Crespo *et al.* 2016), opening the possibility that one or more downstream mediators of Fsh action

stimulate the canonical, β-catenin-dependent pathway. To further investigate this possibility, we examined the effect of three of these mediators, 11-KT (Garcia-Lopez *et al.* 2010), Insl3 (Crespo *et al.* 2016) and Igf3 (Nóbrega *et al.* 2015), on the transcript levels of *cyclinD1* and *axin2*, two genes known to be sensitive to β-catenin signaling also in zebrafish (Neal *et al.* 2013, Wehner *et al.* 2014). In response to Igf3 incubation the transcript levels of both genes increased, while 11-KT and Insl3 were unable to do so (Fig. 1A). Next, testes were incubated in the presence or absence of Fsh or in the presence of Fsh with or without an Igf receptor inhibitor (NVP-AEW541). The transcript levels of *cyclinD1* did not change, while *axin2* transcript increased and decreased in response to Fsh or to Fsh and
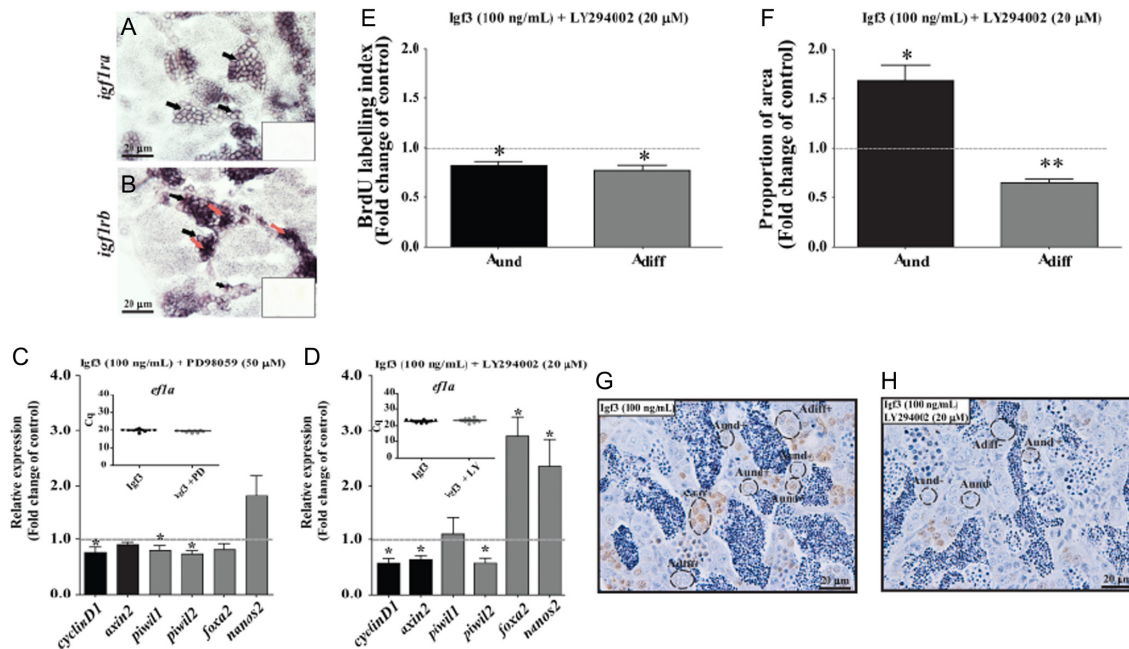
Figure 4

Testicular *igf1 receptor* expression and MAPK and PI3K inhibitor effects on an Igf3-stimulated differentiation of type A spermatogonia. (A and B) Localization of *igf1ra* (A) and *igf1rb* (B) transcripts by *in situ* hybridization using adult zebrafish testis cryosections. Black arrows show staining in SCs and their extension surrounding spermatogonia; red arrows in (B) show staining in spermatogonia. Insets in A and B show the absence of specific labeling when using sense probes. (C and D) Transcript levels of two β-catenin sensitive genes (*cyclinD1* and *axin2*) and germ cell marker genes in the presence of Igf3 (100 ng/mL) alone (the control condition of these experiments, represented by a dotted line) or in the presence of Igf3 in combination with the MAPK inhibitor PD98059 (C; 50 μM; $n = 10$; represented by bars), or the PI3K inhibitor LY294002 (D; 20 μM; $n = 8$; represented by bars). The quantification cycles (Cq) of the reference gene (*elf1a*) are shown in the insets. (E) BrdU-labeling indices of type A_{und} and type A_{diff} spermatogonia in the presence of Igf3 (100 ng/mL; control condition, represented by the dotted line) alone or in combination with the PI3K inhibitor LY294002 (20 μM; $n = 5$; represented by bars). (F) Proportion of section surface area occupied by cysts containing type A_{und} and type A_{diff} spermatogonia in the presence of Igf3 (100 ng/mL; control condition, represented by the dotted line) alone or in combination with the PI3K inhibitor LY294002 (20 μM; $n = 5$; represented by bars). (G and H) Immunocytochemical detection of BrdU in sections of zebrafish testis incubated in the presence of 100 ng/mL Igf3 alone (G) or in combination with the PI3K inhibitor LY294002 (H; 20 μM) for 5 days, showing BrdU-positive (+) and -negative (−) A_{und} and A_{diff} spermatogonia. Results are presented as fold changes with respect to the control group (100 ng/mL Igf3). Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$) to the respective control group that were incubated with Igf3 (100 ng/mL) alone. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0124.

NVP-AEW541, respectively (Fig. 1B). These experiments suggest that Fsh-stimulated Igf3 release activates β-catenin signaling.

To further study the effect of Igf3 on the β-catenin-dependent pathway, testes from *Tg(7xTCF-Xla.Siam:GFP)^ia4* zebrafish were incubated under basal conditions or in the presence of Igf3. A weak signal for Gfp protein was detectable in the absence of Igf3 (Fig. 1C, D and E) but was strongly induced in type A spermatogonia in response to Igf3 (Fig. 1F, G and H). Importantly, an inhibitor of Wnt ligand release (IWP-12) did not modulate the Igf3-mediated induction of Gfp (Fig. 2A, B, C, D, E and F), of the proliferation activity or of the proportion of area occupied by type A spermatogonia (Fig. 2G and H). These experiments show that Igf3-stimulated, β-catenin-mediated spermatogonial proliferation and differentiation was independent of Wnt release. The capacity of IWP-12 to block the release of Wnt ligands

also in zebrafish testis tissue was demonstrated by the IWP-12-induced decrease in Gfp protein levels in tissue from *Tg(7xTCF-Xla.Siam:GFP)^ia4* zebrafish (Fig. 2I and J).

Igf3 uses the β-catenin-dependent pathway to promote differentiation

Previously, we reported that Igf3 increased the proliferation of both types of A spermatogonia and decreased the proportion of A_{und} while increasing the one for A_{diff} spermatogonia (Nóbrega *et al.* 2015, Safian *et al.* 2017), i.e. Igf3 promoted the differentiating proliferation of type A spermatogonia. Here, we examined directly the effect of XAV939 and IWR-1, inhibitors of the β-catenin-dependent pathway, on Igf3-stimulated differentiation of type A spermatogonia. XAV939 and IWR-1 reduced Igf3-stimulated proliferation of both type A spermatogonia to basal levels (Fig. 3A). Regarding the proportion of

area, XAV939 and IWR-1 blocked the pro-differentiation effect of Igf3 on type A_{diff} spermatogonia (Fig. 3B). This suggests that the Igf3-induced transition of A_{und} to A_{diff} was compromised by both inhibitors, associated with a certain accumulation of A_{und} spermatogonia. Immunocytochemical studies using testes of *Tg(7xTCF-Xla.Siam:GFP)ia4* zebrafish showed that Igf3-induced, β-catenin-mediated Gfp protein expression in type A spermatogonia was blocked by XAV939 (Fig. 3C, D, E, F, G and H). Moreover, transcript quantification (yielding reduced *cyclinD1* and *axin2* levels) confirmed inhibition of the β-catenin pathway. Increased transcript levels of type A spermatogonia-marker genes (*foxa2* (Safian *et al.* 2017) and *nanos2* (Beer *et al.* 2013, Bellaiche *et al.* 2014) and decreased transcript levels of marker genes for more differentiated germ cells (*piwil2* (Houwing *et al.* 2008) and *dazl* (Chen *et al.* 2013) (Fig. 3I) are in line with the morphological data (Fig. 3B).

Cellular localization of Igf1 receptors and downstream pathways used by Igf3 to activate β-catenin-dependent pathway

To better understand the mechanism used by Igf3 to stimulate β-catenin-dependent processes, we studied the cellular localization of *igf1 receptors a* and *b* by *in situ* hybridization. Moreover, we tested the effects of a MAPK (PD98059) and of a PI3K (LY294002) inhibitor on the expression of β-catenin-sensitive and germ cell marker genes.

In situ hybridization revealed that *igf1ra* was expressed by SCs contacting all germ cell generations from spermatogonia to spermatids, while *igf1rb* was expressed by both SCs and germ cells (Fig. 4A and B).

The transcript levels of *cyclinD1*, but not of *axin2*, were down regulated by the MAPK inhibitor in Igf3-stimulated testis tissue (Fig. 4C). Concerning germ cell-marker gene expression, the mRNA levels of *piwil1* (expressed by all spermatogonia and primary spermatocytes; Houwing *et al.* 2008) and *piwil2* were slightly reduced (Fig. 4C). The PI3K inhibitor, on the other hand, significantly compromised Igf3-mediated increases of both *cyclinD1* and *axin2* transcript levels (Fig. 4D), increased transcript levels of *foxa2* and *nanos2* but reduced those of *piwil2* (Fig. 4D). Overall, this indicates a shift towards more undifferentiated germ cells when interfering with Igf3 effects by adding MAPK or PI3K inhibitors. The morphological analysis pointed into the same direction (Fig. 4E, F,

G and H). The proliferation activity of both A_{und} and A_{diff} spermatogonia decreased while an increased proportion of A_{und} and a reduced proportion of A_{diff} spermatogonia was recorded in the presence of Igf3 and the PI3K inhibitor. This suggests that in addition to a reduced proliferation of type A spermatogonia, also spermatogonial differentiation was compromised, resulting in an accumulation of type A_{und} spermatogonia and a partial depletion of A_{diff} spermatogonia.

Effect of Igf3 on Sertoli cell proliferation

Since SC proliferation is required for fertility in the adult fish testis (De França *et al.* 2015), we have studied the number of BrdU-positive SCs that were free (i.e. apparently not in contact with germ cells) (Fig. 5A) and that contacted A_{und} (Fig. 5B and C) or A_{diff} spermatogonia (Fig. 5D). This was examined under basal conditions or in the presence of Igf3 (100 ng/mL), with or without the inhibitor of PI3K (LY294002, 20 μM) or the β-catenin signaling inhibitor XAV939 (10 μM). Igf3 reduced the number of proliferating SCs contacting BrdU-negative A_{und}, while it increased the number of proliferating SC contacting BrdU-positive A_{diff} (Fig. 5E). In the additional presence of the β-catenin signaling inhibitor XAV939, the number of proliferating free SC and SCs contacting BrdU-negative A_{und} increased while decreasing the proliferation of SCs contacting BrdU-positive A_{und} (Fig. 5F). Similarly, the proliferation of free SC and SCs contacting BrdU-negative A_{und} increased in the presence of the PI3K inhibitor (Fig. 5G).

Discussion

Fsh stimulates spermatogonial differentiation via androgen production in LCs and also by stimulating the transcript levels and release of Igf3, but not those of igf1, igf2a and b (Nóbrega *et al.* 2015; earlier sometimes referred to as Igf1b) mainly from SCs and of insulin-like peptide 3 (Insl3) from LCs (Assis *et al.* 2016, Crespo *et al.* 2016). Since a previous report indicated that Fsh modulated the transcript levels of Wnt pathway-associated genes but did not regulate the expression of Wnt ligands (Crespo *et al.* 2016), we hypothesized that the β-catenin pathway becomes activated by one or more downstream mediators of Fsh action, potentially in a manner independent of Wnt ligands. Our results show that Fsh-stimulated Igf3 release most likely activated β-catenin signaling in germ cells via *igf1rb*. This occurred in a manner involving the PI3K pathway but did not depend on the release of Wnt ligands.
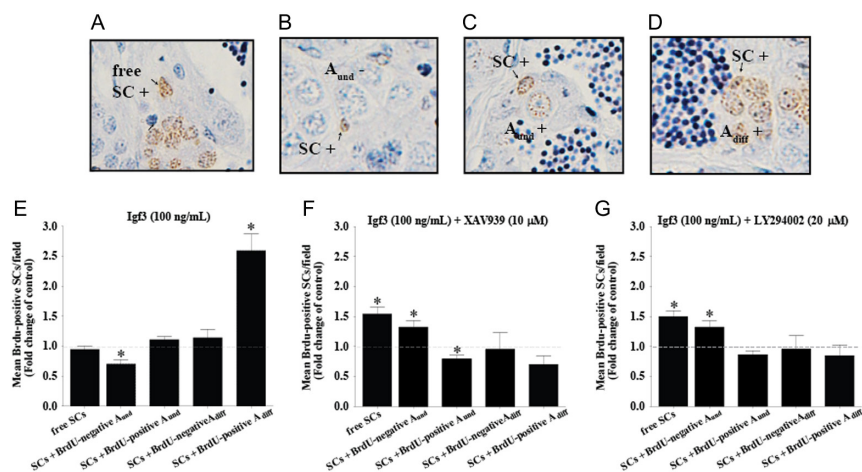
Figure 5
Effect of Igf3 on the proliferation of Sertoli cells contacting type A spermatogonia. Immunocytochemical detection of BrdU in sections of zebrafish testis showing free BrdU-positive SCs (A), BrdU-positive SCs contacting BrdU-negative (−) (B) and -positive (+) (C) A_{und} and contacting BrdU-positive (+) A_{diff} spermatogonia (D). Number of BrdU-positive free Sertoli cells, Sertoli cells contacting BrdU-negative and -positive A_{und}, and Sertoli cells contacting A_{diff} in basal conditions (dotted line) or in the presence of the presence of Igf3 (100 ng/mL; represented by bars; $n=7$) (E) or in the presence of Igf3 (100 ng/mL; dotted line; control condition) with or without 10 µM XAV939 (represented by bars; $n=7$) (F) or 20 µM LY294002 ($n=8$; represented by bars) (G). A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0124.

Activation of β-catenin signaling in type A spermatogonia promoted their differentiating proliferation.

Previous studies have suggested that crosstalk occurs between Igf and the β-catenin pathway in mammals (Playford *et al.* 2000, Desbois-Mounthon *et al.* 2001, Ye *et al.* 2010, Wang *et al.* 2010, Hu *et al.* 2012), but somewhat inconsistent results have been found. Desbois-Mounthon *et al.* (2001), using human liver-derived HepG2 cells, and Wang *et al.* (2010), using murine chondrocytes, proposed that IGF1 uses the PI3K/AKT pathway to inhibit glycogen synthase kinase 3β (GSK3β), an important subunit of the β-catenin destruction complex. Ng *et al.* (2009), on the other hand, proposed that core proteins of the β-catenin destruction complex (in particular axin) protect GSK3β from the inhibitory effect of IGF/PI3K/AKT pathway and that Wnt-mediated transcriptional activity was not modulated by IGF/PI3K/AKT in prostate cancer (PC3) and breast cancer (BT549) cell lines. These inconsistencies may be related to the different experimental approaches and cell lines used. To our knowledge, the present study is the first to propose that a growth factor not belonging to the Wnt family activates the β-catenin-dependent pathway in the testis. Our data also support the view of a stimulatory role of PI3K in this activation.

The fact that Igf3 does not require Wnt ligands to activate β-catenin signaling is a feature not addressed previously in mammals. Porcupine and Wntless (GPR177 in mice) are proteins required for the intracellular transport and release of Wnt ligands (Herr *et al.* 2012). Surprisingly, loss of Gpr177 either in germ cells or SCs is compatible with normal spermatogenesis in mice and only after 8 months of age, germ cell-specific *Gpr177* knockout mice showed smaller testis and reduced fertility (Chen *et al.*

2016). However, the latter study did not examine the state of activity of the β-catenin-dependent pathway in *Gpr177* knockout mice. We found previously that Igf3 promoted the proliferation of both type A spermatogonia and stimulated the accumulation of A_{diff} while reducing the proportion of A_{und} (Nóbrega *et al.* 2015, Safian *et al.* 2017). Here, we report that blocking the β-catenin-dependent pathway inhibits in particular the pro-differentiation effects of Igf3 in zebrafish spermatogonia. A possible explanation for the absence of a clear and immediate effect of the loss of Wnt ligand release on spermatogenesis in mice may be related to our finding that signaling molecules of other pathways, such as Igf ligands, can stimulate β-catenin-dependent signaling in a Wnt ligand-independent manner in germ cells.

As mentioned earlier, Igf3 activated β-catenin signaling in type A spermatogonia. However, information on the cellular localization of Igf receptors in the zebrafish testis was missing. In rainbow trout, *igf1r* expression was found in cell fractions enriched in SCs but also in spermatogonia and primary spermatocytes (Le Gac *et al.* 1996). We report here that both Igf receptors were expressed by SCs but that *igf1rb* was also expressed by zebrafish germ cells. This opens the possibility that Igf1rb mediates the Igf3 effects to activate the β-catenin-dependent pathway in spermatogonia. Our results also suggest that Igf3 uses PI3K, and not MAPK, to activate β-catenin-dependent signaling. Similarly, Wang *et al.* (2014) reported that IGF1 stimulated the proliferation of murine spermatogonial stem cells in culture via PI3K and not via MAPK signaling. The PI3K pathway has been implicated previously in modulating spermatogonial proliferation in mammals. Shen *et al.* (2014) reported that IGF1 activated PI3K and

MAPK pathways to stimulate differentiation of germ cells in culture of mice testicular fragments. The present study is the first to report that PI3K and β-catenin pathways interact in testis tissue; however, further investigation will be require to elucidate how exactly the PI3K pathway is involved.

Type A_{und} spermatogonia are single germ cells enveloped by one or two SCs (Leal *et al.* 2009). This germ cell population also includes the SSCs in the zebrafish testis (Nóbrega *et al.* 2010). Self-renewing SSCs produce two independent A_{und} daughter cells, each of them associating with SCs that support their survival to form new spermatogenic cysts. Consequently, the production of new spermatogenic cysts also requires the availability of 'free' SCs. When going through a differentiating division, on the other hand, a single A_{und} cell produces two smaller A_{diff} spermatogonia that remain connected via a cytoplasmic bridge within their spermatogenic cyst formed by SCs enveloping the developing germ cell clone. When A_{diff} spermatogonia continue to go through differentiating mitotic cell cycles, germ cell number and hence volume increases, which also requires SC proliferation. Since for both, the formation of new cysts and the developmental growth of existing cysts, SC proliferation is required in the adult testis (De França *et al.* 2015), we have also studied the proliferation of Sertoli cells under the influence of Igf3 and the additional presence of β-catenin signaling or PI3K inhibitors. Igf3 reduced the number of BrdU-positive SC contacting BrdU-negative A_{und}, suggesting a reduced level of activity of the earliest stage of spermatogonial cysts. On the other hand, we recorded a clear increase in the number of BrdU-positive SC contacting BrdU-positive type A_{diff} spermatogonia. Since this Igf3-mediated stimulation of SC proliferation was independent of PI3K and β-catenin signaling, we suspect it was mediated by the Igf receptors localized on SCs. Interestingly, this Igf3-mediated increase of SC proliferation was observed only in cysts where also the A_{diff} spermatogonia had incorporated BrdU, suggesting that the effect potentially involves germ-Sertoli cell communication to coordinate SC and germ cell proliferation. Overall, Igf3 seems to support the further growth of existing spermatogenic cysts, i.e. facilitates differentiation.

In conclusion, we have shown that Fsh-triggered Igf3 release stimulates β-catenin-signaling in a manner involving PI3K signaling, and that this pathway operates independent of Wnt ligands but is required to promote differentiation of A_{und} spermatogonia in zebrafish testis (Fig. 6).

Figure 6
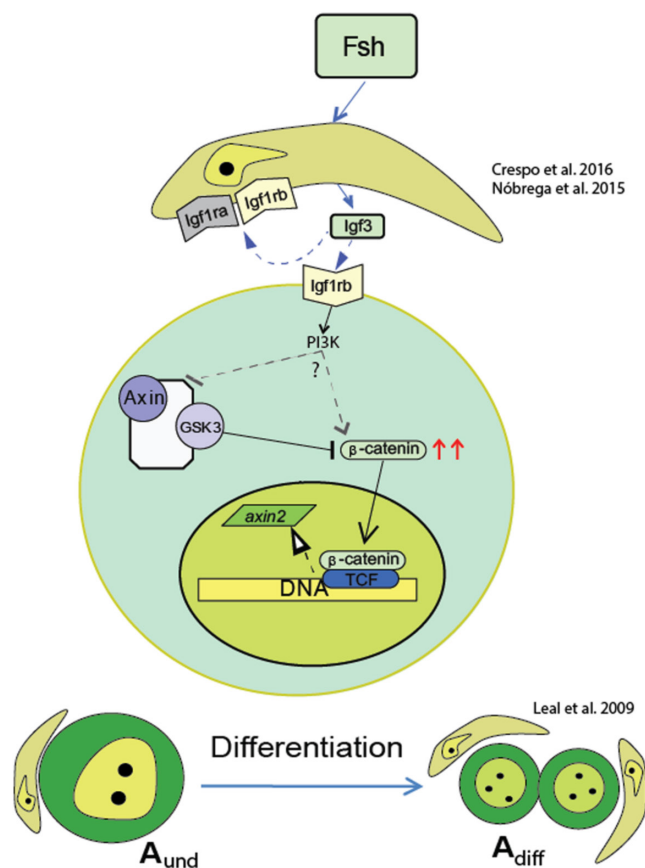Schematic representation of the effects of Igf3 on spermatogenesis by modulating canonical Wnt signaling. Fsh-stimulated Igf3 stimulates differentiating divisions of spermatogonia by increasing Igf3 release from Sertoli cells, which activates β-catenin-dependent, canonical Wnt signaling. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0124.

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